Expression of Ca-Binding Protein Recoverin in Normal, Surviving, and Regenerating Retina of *Pleurodeles Waltl* Adult Triton

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Immunohistochemical study of the expression of recoverin (photoreceptor protein) in the retina of *Pleurodeles waltl* adult triton was carried out in health, during regeneration after removal, and under conditions of long-lasting detachment. Studies with polyclonal (monospecific) antibodies to recoverin showed that normally it is present in the internal segment, connective cilium, in distal portions of the external segments of cones and rods, and in Landolt clubs of displaced bipolar cells. Detachment of the retina is associated with translocation of recoverin from the photoreceptor processes to perikaryons, and the content of recoverin-positive displaced bipolar cells increases. During regeneration of the retina after its excision via conversion of the pigmented epithelial cells, recoverin is synthesized in the prospective photoreceptor perikaryons and then accumulates in the forming inner segments. Hence, recoverin can serve as a reliable marker in studies of photoreceptor differentiation and functioning during regeneration or survival of the retina.

Key Words: *immunohistochemistry; triton; retina; regeneration; recoverin*

Recoverin (calcium-binding protein) [3,11,20] was detected in the retinal photoreceptors in animals of different classes [10] and in humans [5,31]. The data on the localization of recoverin and its homologues in *Urodela* retina are scanty: the findings of our previous study on *Pleurodeles waltl* [1] and detection of recoverin in tiger salamander photoreceptors [23,33]. Special interest to studies of recoverin expression in tritons is explained primarily by unique capacity of these animals to regeneration of the retina after its excision or after crossing of the optic nerve [15,22] and to regeneration after long-lasting detachment of the retina from the pigmented epithelium [2]. In tritons, the retina regenerates after removal at the expense of pigmented epithelial cells and the growth zone of the eye. Transdifferentiation, proliferation, and subsequent neurogenesis processes underlie this regeneration [9,22]. After detachment from the pigmented epithelium leading to death of some cells, the retina in tritons regenerates with the involvement of Mueller glial cells, precursors of vitreal row cells of the inner nuclear layer of the retina, and displaced bipolars with Landolt clubs [2,14].

Antibodies to opsins are traditionally used in studies on triton retina as specific markers of photoreceptors [6,24,26]. However, recoverin is specific of vertebrate retinal photoreceptor cells. It was not clear whether this protein could serve as the marker of photoreceptor cells and their precursors during regeneration of the retina after its removal and partial destruction as a result of detachment from the pigmented epithelium. It was also essential to evaluate the pattern of this protein distribution in the photoreceptors, depending on the development (differentiation) of

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photoreceptors and under conditions of their dissociation with pigmented epithelium cells in detachment of the retina.

We previously showed that antibodies to recoverin detect the only protein band with a molecular weight about 26 kDa coinciding with that of recoverin in adult *Pl. waltl* retina protein extract [1]. Using polyclonal (monospecific) antibodies to recoverin, we carried out an immunohistochemical study of the location of this protein in *Pl. waltl* retina in health (*in situ*) and during regeneration of the retina after its detachment and after its complete surgical excision.

MATERIALS AND METHODS

Immunohistochemical study. Polyclonal monospecific antibodies to recoverin were obtained as described previously [3,11] by immunization of rabbits with recombinant myristoyl-treated recoverin [27]. Before immunohistochemical study, the antibodies to recoverin were diluted to a final concentration of 0.011 mg/ml in 0.1 M phosphate buffer (pH 7.4; buffer A) with 0.3% Triton X-100 and 0.5% normal goat serum or 0.1% BSA. Immunohistochemical study was carried out by the indirect immunofluorescent method or immunoperoxidase staining on paraffin and frozen sections of the eyes from adult *Pl. waltl* obtained from aquarium of Institute of Developmental Biology. The eyes were enucleated in anesthetized animals in MS 222 water solution (1:1500, Sigma). This procedure and all subsequent manipulations were carried out in accordance with regulations of the Russian Academy of Sciences for studies on amphibians. The preparations were fixed in formalin or in ethanol supercooled in liquid nitrogen. In the former case, the eyes were plunged in 4% formalin in buffer A for 12 h at 4°C, washed (3×15 min) in 5% sucrose solution in buffer A, impregnated overnight in 20% sucrose solution in the same buffer, frozen in Jung medium (Leica), and 10-μ sections were sliced on a cryostat. If ethanol was used, the preparations were fixed for 2 days at -60°C and then at ascending temperatures (-20, -4, 4°C; 24 h at each temperature). The resultant preparations were washed in buffer A at 4°C, dehydrated once more, and embedded in paraplast; 7-μ sections were sliced.

Radial and tangential sections of different retinal areas from normal and operated on eyes were examined. The sections were washed in buffer A, incubated in buffer A with 3.3% normal goat serum or 0.1% BSA (30 min), after which were washed once more. Sections for immunoperoxidase staining were additionally treated with 0.014% H_2O_2 for endogenous peroxidase binding, after which they were washed in buffer A. Then the sections were incubated with antibodies to recoverin (diluted 1:20) overnight at 21-23°C. After incubation, the sections were washed and incubated with second (rabbit FITC-conjugated) antibodies (Sigma) diluted 1:150 or with HRP-conjugated antibodies (Sigma) diluted 1:100. For this latter case, the sections were again washed and staining was developed for 5 min in aqueous solution of DAB+H_2O_2 (Sigma). The sections were then washed, dehydrated, and embedded in Canadian Balm under coverslips. The sections stained with FITC-conjugated antibodies were washed and embedded in Vectashield medium (Vector).

Control sections stained with only second (FITC- or HRP-conjugated) antibodies demonstrated the absence of immunospecific reaction, which was also seen in additional controls with the DAB+H_2O_2 developing solution. Immunofluorescence was analyzed in an MC80DX Zeiss or in Olimpus AH-3 microscope. The pictures were taken with a digital camera and processed using Lite software.

Isolation and dissociation of triton retinal photoreceptors. The eyes were plunged in 20% sucrose solution in buffer A, the posterior segments were isolated, and plunged in the tubes with dissociating medium. Ten-minute collagenase treatment with periodical shaking at ambient temperature proved to be the best of the photoreceptor dissociation methods (collagenase, EDTA, or trypsin treatment with subsequent centrifugation and washing). The solution was prepared by mixing equal volumes of 0.1% collagenase (Fluka) solution in buffer A and 20% sucrose solution in the same buffer. The resultant cell suspension was pipetted, dried, washed in buffer A, fixed on slides with 4% formalin solution in this buffer, and washed again. The resultant preparations with external segments of rods, few whole photoreceptors, and bipolar cells with Landolt clubs were immunohistochemically stained for recoverin as described above. Specimens of triton regenerating retina were dissociated and processed in the same way.

Experimental detachment of the retina. Detachment of the retina from the pigmented epithelium was carried out as described previously [2]. Animal eyes were fixed 5, 10, 15, 20, 30, 40, or 60 days after the operation. Immunohistochemical study of eye sections from operated tritons was carried out similarly as of intact triton eyes, with FITC- and HRP-conjugated antibodies serving as the second antibodies.

Study of recoverin expression in triton regenerating retina. After narcotization, the retina was excised by Stone's method [29] from both eyes of adult tritons. The eyes were enucleated in narcotized animals 10, 15, 20, 30, and 40 days after the operation. The eyes were fixed, washed, and frozen as described above. Radial sections (paraffin after ethanol fixation and frozen after formalin fixation) from the periphery and center of the retina were sliced from 4

eyes for each term postoperation. Immunohistochemical staining of the sections was carried out. The retinal regeneration stages were evaluated according to the classification by C. Chiba *et al.* [8].

RESULTS

Recoverin in normal *Pl. waltl* retina. On the whole, the results of recoverin location in normal triton retina coincided with our previous findings [1]. Recoverin-positive staining in tangential sections was seen only in the external retina, while the internal part of the retina, growth area (*pars ciliaris — ora serrata*), and pigmented epithelium of the retina were recoverin-negative. The internal segments of rods and cones exhibited the brightest fluorescence in the photoreceptor layer. Faint fluorescence in structures differing from the internal segments was seen close to them at the level of the outer borderline membrane. Areas of slight expression of recoverin coincided with terminals of the main dendrites (Landolt clubs) of displaced bipolar cells of the outer nuclear layer. Analysis of immunofluorescence in the radial sections of triton retina sliced strictly along the photoreceptor segments also showed staining in the internal segments of both photoreceptor types (Fig. 1). At high resolution (×1000), staining of the connective cilium and basal part of the external segments of photoreceptors and faint recoverin-specific fluorescence of Landolt clubs was detected.

Recoverin in dissociated cells of normal triton retina. Dissociated photoreceptors were stained with antibodies to recoverin similarly as the retinal photoreceptors *in situ*. Immune reaction was clearly seen in the internal segments, connective cilium, and basal parts of external segments in the rods and cones. Slight fluorescent staining of the external segments of rods could be detected in isolated preparations (Fig. 2, *a*). Dendrites and less so the cell body and axon were stained in isolated displaced bipolar cells (Fig. 2, *b*).

Expression of recoverin in triton retina after detachment from the pigmented epithelium. The total number of recoverin-positive cells decreased during the early (5 days) period after surgery. Photoreceptors in the newly formed retinal folds lost their immunoreactivity. The pattern of staining was unchanged in the photoreceptors retaining the immune response to antibodies to recoverin. In addition, the dendrites (Landolt clubs) in displaced bipolar cells were stained. The most characteristic sign of later (days 10-15) period was development of specific staining in photoreceptor bodies, in the cytoplasm around their nuclei, where the immunofluorescence intensity was higher than in internal segments (Fig. 3). Hence, the immunoreactive material was redistributed from the proximal to distal compartments of the cell during this period. This picture was observed during 20-30 days postoperation. During the same period, the number of recoverin-positive displaced bipolar cells increased, presumably, at the expense of additional migration of this subpopulation from the internal nuclear layer. Later (days 40-60), the percentage of cells with normal pattern of recoverin expression increased with further recovery of the cellular composition of triton retina.

Development of recoverin expression in triton retina during its regeneration after excision. Pigmented epithelium cells dedifferentiated and proliferated with the formation of 2-3 rows of partially pigmented cells during regeneration of triton retina in the early (days 10-15) period postoperation, stages E1—E3). There were no recoverin-positive cells in the regenerating tissue (Fig. 4, *a*). No recoverin expression was detected in dissociated cells collected during this stage. Later (on day 20 postoperation; stage I-1), the number of arbitrary rows of regenerating tissue cells increased to 4. Solitary recoverin-positive cells appeared in the regenerating tissue near the pigmented epithelium and only in places where the primordium

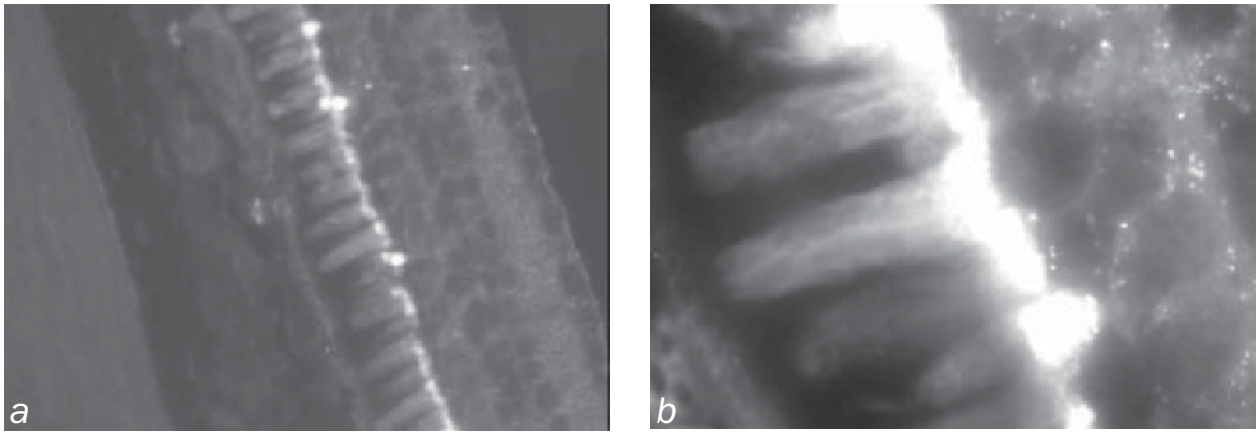

Fig. 1. Recoverin location in normal triton retina: recoverin expressed in internal segments of rods and cones. *a*) ×200; *b*) ×1000.

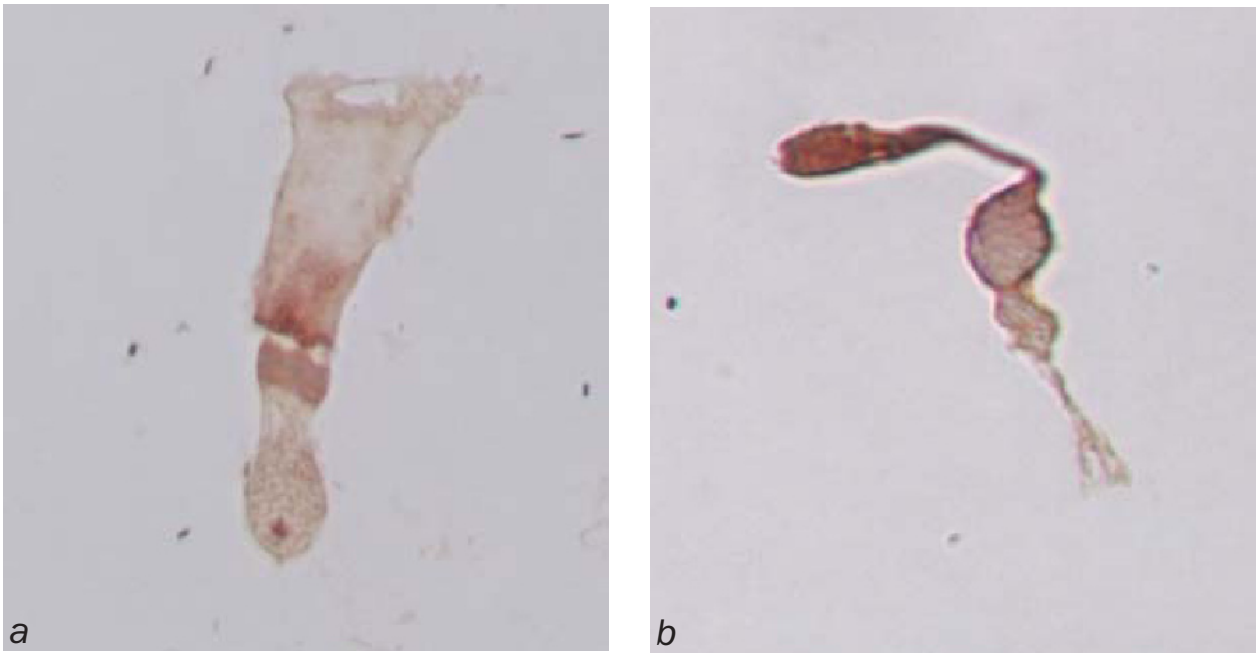

Fig. 2. Recoverin location in isolated cells of external nuclear layer of triton retina: in the rod (*a*) and displaced bipolar with Landolt club (*b*). HRP staining (×1000).

was the thickest by this period (Fig. 4, *b*). During this period recoverin-positive cells were not yet definitely differentiated and were evenly stained throughout their entire surface, which was particularly well seen in tangential sections (Fig. 5, *a*). During the intermediate stage (days 20-30 postoperation, stage I-2), the regenerate consisting of actively proliferating and morphologically undifferentiated cells was growing. The number of recoverin-positive cells on the outer surface of the regenerate increased with increasing in the count of its cells (Fig. 4, *c*). Separate cells formed the internal segment presented by a formation protruding on the proximal surface of the future photoreceptor (Fig. 5, *b*). The intensity of recoverin-specific staining in this compartment was notably higher than around the body of the cell. Differences in the count and distribution of recoverin-positive cells were detected, depending on the degree of primordial retina development, its thickness and level of differentiation. Stratification of the retinal regenerate and differentiation of its cells started during the late intermediate stage (day 30 postoperation, stage I-3): the ganglion level and primordial retinal layers were forming. During this period all prospective photoreceptors in the best differentiated retinal areas became recoverin-positive, though their staining differed from that of mature cells: the bodies of the cells and their axons were still stained (Fig. 4, *d*; 5, *c*). Individual recoverin-positive cells were detected in the internal rows of the regenerate cells during this stage. Judging from the location of these cells, they could be referred to prospective displaced bipolar cells. The late stage (day 40 postoperation, stage L-1) was characterized by differentiation of the nuclear and reticular layers of the retina. Recoverin-positive cells during this stage constituted a vast outer nuclear layer, but the pattern of staining of individual photoreceptors still differed from the normal picture. Presumably, this was explained by still incomplete differentiation of photoreceptor cells and formation of contacts with the pigmented epithelium cells (Fig. 5, *d*).

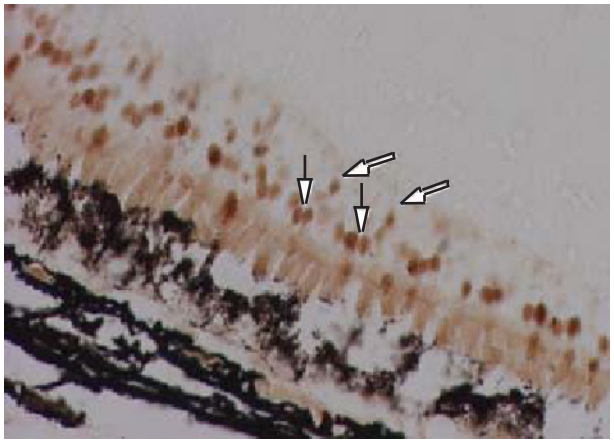

Fig. 3. Recoverin location in external nuclear layer of retina on day 15 after detachment from the pigmented epithelium: staining of photoreceptor cell perikaryons (double arrows) and displaced bipolars (arrows). HRP staining (×200).

Recoverin, first detected in the retina of higher vertebrates [3,11,20], is assumed to serve as Ca^{2+} sensor of rhodopsin phosphorylation in the photoreceptor

external segments [12,13,18,19]. Bovine antibodies to recoverin are capable of cross-reacting with triton recoverin [1] and with recoverin ortholog S-modulin present in frog retina [17,18]. Since amphibians are an animal class far from mammals, we can speak about highly conservative nature of recoverin. Other photoreceptor proteins are also highly conservative, for example, rhodopsin, arrestin, transducin α-subunit, and cGMP phosphodiesterase [25]. According to our data (the present and previous [1] findings), the pattern of recoverin distribution in normal triton retinal cones and rods somewhat differs from that in the majority of mammals. In mammalian photoreceptors, recoverin is present mainly in the external, while in triton it is detected mainly in the internal segments of photoreceptors. However, in some mammals, for example, in squirrels and mice, recoverin location is similar to that in *Urodela* [28,30]. Recoverin expression in the retinal photoreceptor internal segment of lower and some higher vertebrates can be due to the synthesis of this protein (similarly as of other optic proteins) in this cell compartment [6,16]. It is also possible that these differences are explained by modifications of methods for preparation of the material and availability of the antigen. In order to verify this hypothesis, we additionally studied the location of recoverin in retinal dissociated cells. The results were similar to those obtained *in situ*: immunoreactivity was still low in external segments of photoreceptors and increased only in the axoneme (site of photoreceptor disk forma-

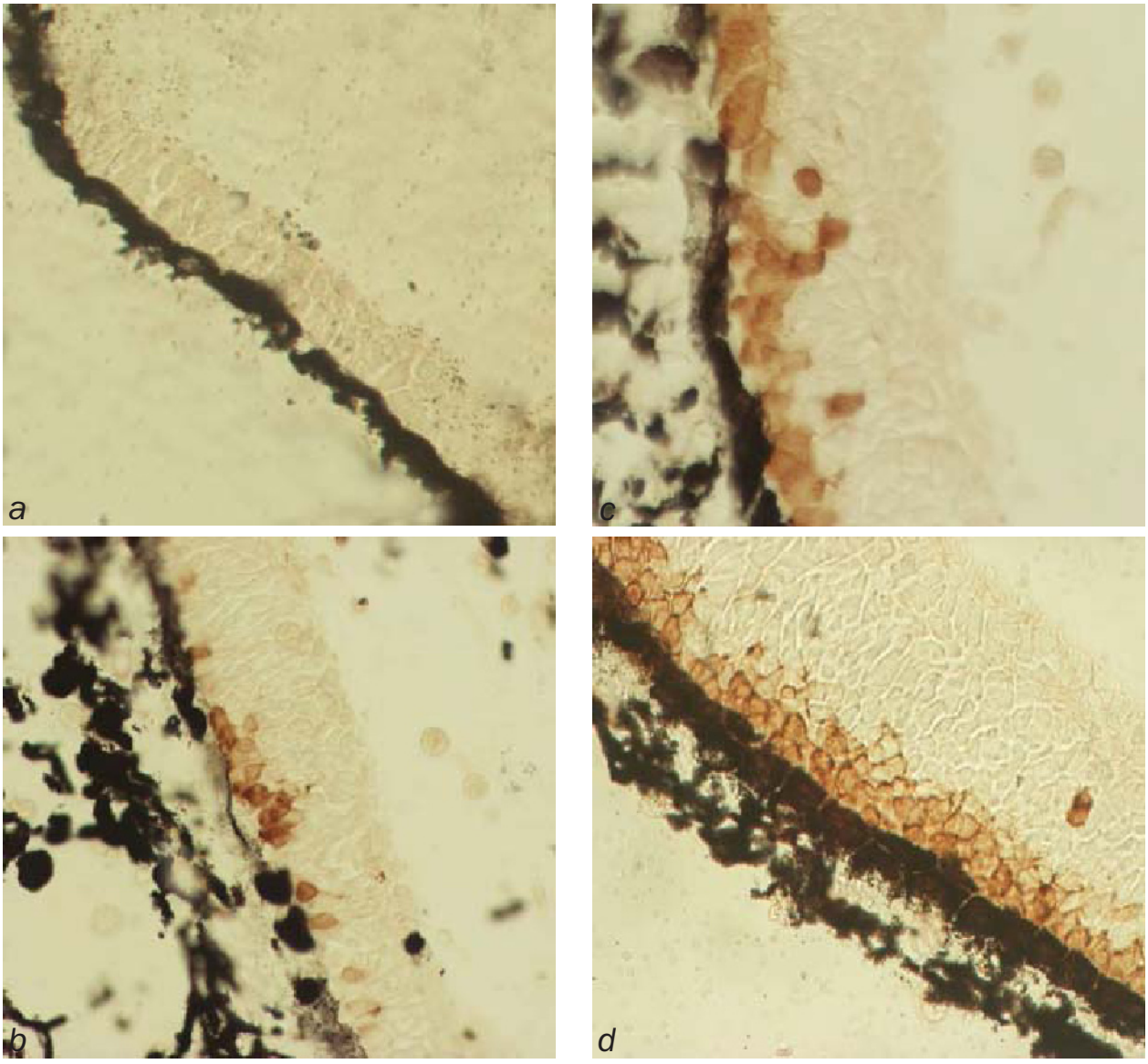

Fig. 4. Expression of recoverin at different stages of retinal regeneration after its excision. *a*) early stage (E3); *b*) intermediate stage (I-1); *c*) late intermediate stage (I-2); *d*) late regeneration stage (L-1). HRP staining (×200).

tion and active protein transport). On the other hand, recoverin is expressed in segments and bodies of cones and rods in the tiger salamander retina [33]. Hence, it is most possible that recoverin distribution in *Urodela* retinal photoreceptor cells is not species-specific, but reflects the capacity of this protein to physiological transposition in photoreceptor cells in these animals.

Our findings also indicate slight recoverin-positive immune reaction in the main dendrites (Landolt clubs) of displaced bipolars. Definitely stained Landolt club in cell dissociation and less intensely stained body of the cell indicate the presence of recoverin in dendrites of these cells, analogous to the photoreceptor internal segment. This indicates dual type of differentiation of these cells and confirms the hypothesis on the possibility of their recruiting for performing the photoreceptor function [2,14].

Changes in recoverin location were detected in experimental detachment of the retina in triton. Recoverin was redistributed from apical to distal compartments, into photoreceptor bodies on days 10-15 postoperation and remained there until recovery of photoreceptor interactions with pigmented epithelium cells. Recoverin translocation into the bodies of cells was demonstrated on cultured retinal explants from triton [4] and from postnatal rats [21]. The authors explained this phenomenon by continuing biosynthesis of recoverin in the cell cytoplasm and disorders in

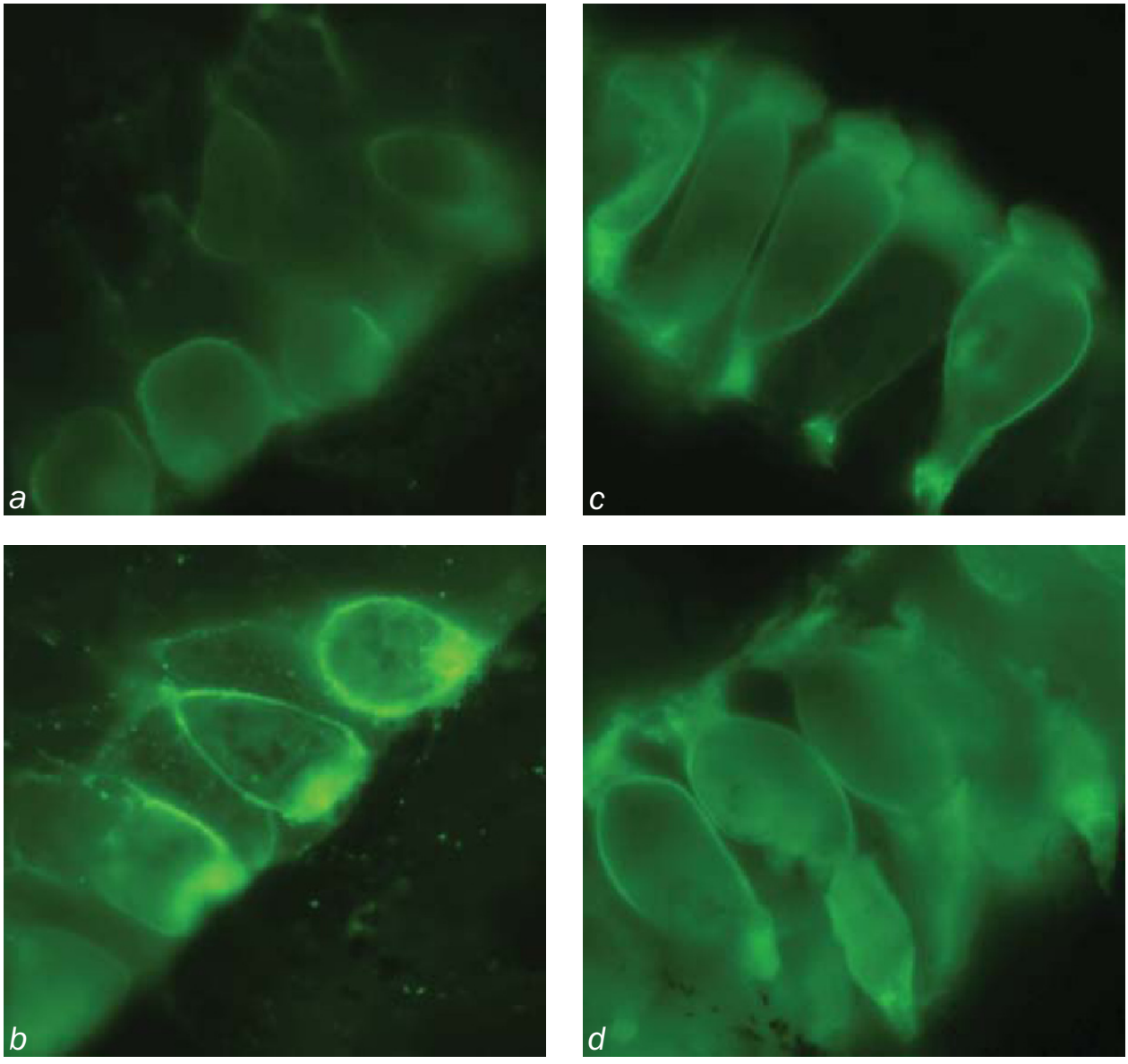

Fig. 5. Recoverin location in forming photoreceptors at different stages of retinal regeneration after excision (×1000). *a*) in undifferentiated prospective photoreceptors; *b*) in cells starting to form the internal segment; *c*) in cells forming the external segment; *d*) in young differentiated photoreceptors.

the regulation of translocation of newly synthesized molecules. The changes in recoverin location in triton retina under conditions of its detachment observed in our experiments can be due to the same causes, which, in turn, result from disorders in interactions of retinal photoreceptors with pigmented epithelial cells.

Analysis of recoverin expression development in new (emerging after excision of the initial) retina showed that transdifferentiated pigmented epithelium cells (sources of regeneration) remained recoverin-negative during the early stages. The same is known about opsins, studied on the same model, but in other triton species [6,26]. Discussing the mechanism initiating recoverin expression during regeneration of the retina, we can hypothesize that the gene encoding recoverin is silent in pigmented epithelium cells (sources of regeneration), but during transdifferentiation of pigmented epithelium cells the recoverin gene promotor is demethylated, which triggers its activity. When the number of the retinal regenerate cell rows reaches five, the descendants from the pigmented epithelium, first perinuclearly stained recoverin-positive cells, still without signs of morphological differentiation, appear near it. Later (at the beginning of the regenerate stratification) the number of recoverin-positive cells on the external surface of the primordial retina sharply increases and the internal segments forms. Analysis of opsin expression during photoreceptor formation in the regenerating retina of *Cynops pyrrhogaster* triton also showed the appearance of these proteins first in bodies of cells and then in the external compartments [26]. A similar order of expression development during regeneration of the retina was observed in experiments on adult *Triturus pyrrhogaster* tritons for visinin, a Ca^{2+}-binding protein related to recoverin [24]. During differentiation of the retina, visinin expression increases in some postmitotic cells (prospective cones) of the regenerating retina, similarly as recoverin expression during regeneration of the retina in *Pl. waltl*. Hence, the vector of redistribution of recoverin and some other photoreceptor proteins, studied in this respect, is opposite during photoreceptor differentiation in regeneration of the retina in comparison with detachment of the retina and cell culturing.

We did not study the impact of illumination of the retina for recoverin location in photoreceptors in these experiments. However, we previously detected light-dependent translocation of recoverin and other photoreceptor proteins [7,32]. We found recoverin translocation phenomenon in triton and showed that it can also take place in regeneration/development of the retina and under conditions of its detachment from the pigmented epithelium. It is noteworthy that recoverin can be regarded as the earliest marker of photoreceptor differentiation in tailed amphibians (animals widely used in studies of retinal regeneration and physiology). The appearance of other photoreceptor proteins (opsins and even their RNA) was detected somewhat later, at the beginning of synaptogenesis and stratification of the regenerate [6,16,24,26]. In addition, these data suggest that recoverin can be used as a marker of subpopulation of Landolt club bipolar cells, retaining its photoreceptor potential and capable (in the *Pl. waltl*) of replenishing the photoreceptor pool after death of some destroyed cells.

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